Multiple Endocrine Neoplasia Type 1 Interacts with Forkhead Transcription Factor CHES1 in DNA Damage Response

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Abstract
Multiple endocrine neoplasia type 1 (MEN1) is a cancer susceptibility syndrome affecting several endocrine tissues. Investigations of the biochemical function of the MEN1 protein, menin, have suggested a role as a transcriptional co-modulator. The mechanism by which MEN1 inactivation leads to tumor formation is not fully understood. MEN1 was implicated to function in both regulation of cell proliferation and maintenance of genomic integrity. Here, we investigate the mechanism by which MEN1 affects DNA damage response. We found that Drosophila larval tissue and mouse embryonic fibroblasts mutant for the MEN1 homologue were deficient for a DNA damage-activated S-phase checkpoint. The forkhead transcription factor CHES1 (FOXN3) was identified as an interacting protein by a genetic screen, and overexpression of CHES1 restored both cell cycle arrest and viability of MEN1 mutant flies after ionizing radiation exposure. We showed a biochemical interaction between human menin and CHES1 and showed that the COOH terminus of menin, which is frequently mutated in MEN1 patients, is necessary for this interaction. Our data indicate that menin is involved in the activation of S-phase arrest in response to ionizing radiation. CHES1 is a component of a transcriptional repressor complex, that includes mSin3a, histone deacetylase (HDAC) 1, and HDAC2, and it interacts with menin in an S-phase checkpoint pathway related to DNA damage response. (Cancer Res 2006; 66(17): 8397-403)

Introduction
Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant cancer susceptibility syndrome affecting multiple endocrine organs, particularly parathyroid, pancreatic islets, and pituitary (1).

The MEN1 gene localizes to chromosome 11q13 and encodes a 610–amino acid nuclear protein, menin (2–5). Investigation of the biochemical function of menin suggested a possible role as a transcriptional co-modulator. Menin was shown to interact with and inhibit the activity of the activator protein-1 (AP-1) transcription factor component JunD (6) as well as other transcription factors, such as nuclear factor-κB, Smad3, and Pem (7–9). Menin also associates with histone deacetylase (HDAC; refs. 10, 11) and histone methyltransferase (12–15) complexes, which suggests that MEN1 might regulate transcription through the recruitment of chromatin-modifying enzymes.

Menin has been implicated in both regulation of cell proliferation and maintenance of genomic integrity. Overexpression of menin repressed proliferation and tumorigenesis of Ras-transformed NIH3T3 and insulinoma cells (16). Data are conflicting on whether menin-deficient mouse cells have increased proliferative capacity. One study found no cell-autonomous proliferative defects in Men1−/− embryonic stem cells or in mouse embryonic fibroblasts (MEF; ref. 17). However, another study showed that Men1−/− MEFs had increased proliferative capacity, and reintroduction of wild-type (WT) menin was shown to suppress their growth possibly through an interaction with activator of S-phase kinase (ASK), a component of the Cdc7/ASK kinase complex (18). In addition, tissue-specific inactivation of MEN1 in pancreatic islet cells of the mouse leads to widespread hyperplasia (19).

On the other hand, several investigations implicated menin to function in the maintenance of genomic integrity. The lymphocytes from MEN1 patients (heterozygous for MEN1 mutation) as well as menin-deficient MEFs are hypersensitive to the cross-linking agent diepoxybutane and exhibit an elevated frequency of chromosomal abnormalities after the exposure to this agent (20, 21). Menin interacts with FANCID2, one of the genes mutated in the genetic instability syndrome Fanconi anemia. This interaction is strengthened after the exposure of cells to γ-irradiation (21). Interestingly, the hallmark of Fanconi anemia is sensitivity to DNA cross-linking agents. We have recently reported that inactivation of the MEN1 homologue in Drosophila (Mnn1) resulted in flies that were hypermutable as well as hypersensitive to ionizing radiation and DNA cross-linking agents (22). Menin also interacts with replication protein A (23), a protein that binds ssDNA and is important for several DNA repair pathways (24). These data support the role of MEN1 in the maintenance of genome stability.

In the present work, we investigate the integrity of DNA damage checkpoints in Mnn1 mutant Drosophila and in MEFs deficient for MEN1. We also describe a biochemical and genetic interaction between menin and a forkhead/winged helix transcription factor, CHES1 (FOXN3), originally identified for its ability to suppress DNA damage sensitivity phenotype in several checkpoint-deficient yeast strains (25). Our data indicate that menin and CHES1 cooperate in a DNA damage response pathway.

Materials and Methods
G2-M arrest in Drosophila larval tissue. Wing and eye imaginal discs were dissected from the third instar larvae at various time points after 2 krad of ionizing radiation. Discs were fixed in 4% paraformaldehyde in PBS and stained with rabbit phosphorylated histone H3 antibody (Upstate, Charlothesville, VA) followed by FITC-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA). The number of mitotic cells after irradiation was counted under a confocal microscope. At least 10 discs of each type were scored for each time point.
S-phase arrest in Drosophila larval tissue. Larvae were collected 110 to 120 hours after egg deposition, washed in PBS, and irradiated in a 137Cs irradiator to a dose of 4 krad. One hour after irradiation, brains were dissected in PBS. Bromodeoxyuridine (BrdUrd) staining was done as described (26).

Irradiating radiation sensitivity and S-phase arrest in MEFs. WT MEFS were a gift from the laboratory of Dr. Tony Koleske (Yale University, New Haven, CT). Men1+/− MEFs were a gift from the laboratory of Dr. Xiaxin Hua (University of Pennsylvania, Philadelphia, PA; ref. 21). Cells were grown in DMEM (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 100 μg/mL streptomycin, 100 units/mL penicillin, 1% nonessential amino acids, and 1% l-glutamine. For Sigma, St. Louis, MO), and 400 μm L-glutamine.

Ethanol, washed with PBS, and treated with 100 μL of propidium iodide staining 12 hours after irradiation, cells were fixed in 70% ethanol, 6-diamidino-2-phenylindole (DAPI) to visualize nuclei. For propidium iodide staining, exponentially growing cells were seeded into 100-mm plates and irradiated to indicated doses 24 hours later in 137Cs irradiator. Seven days after irradiation, cells were fixed with methanol and stained with crystal violet. For BrdUrd staining, exponentially growing MEFS were irradiated as described above. Twelve hours after irradiation, medium was exchanged for DMEM plus 10 μL/mL BrdUrd (Amersham, Piscataway, NJ) for 15 minutes. Cells were washed with ice-cold PBS, trypsinized, and fixed with 70% ethanol, and their DNA was denatured by 2 N HCl, neutralized with PBS plus 0.3% Triton X-100, washed in PBS plus 0.5% Tween 20 plus 2% bovine serum albumin, and hybridized to FITC-conjugated anti-BrdUrd antibody (Becton Dickinson, San Jose, CA). Cells were analyzed for FITC fluorescence using a BD FACSCalibur four-color analysis cytometer (Becton Dickinson), and data were analyzed with CellQuest software. For fluorescent microscopy analysis, a similar procedure was followed with the exception that cells were grown on coverslips, were not trypsinized, were fixed with 5% formaldehyde, and were stained with 0.2% 4′,6-diamidino-2-phenylindole (DAPI) to visualize nuclei. For propidium iodide staining 12 hours after irradiation, cells were fixed in 70% ethanol, washed with PBS, and treated with 100 μL RNase (100 μg/mL; Sigma, St. Louis, MO), and 400 μL of propidium iodide solution (50 μg/mL in PBS). DNA content analysis was done on a FACSscan three-color analysis cytometer, and data were analyzed using Flojo software.

Cell culture conditions, transfections, and immunoprecipitations. HEK293 cells were grown in DMEM supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 units/mL penicillin. Cells were transfected using the Fugene 6 reagent (Roche, Penzberg, Germany) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were lysed with radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% NP40, 0.25% Na-deoxycholate] supplemented with a tablet of complete protease inhibitors (Roche). 50 mmol/L NaF, and 1 mmol/L Na3VO4 (Sigma). Lysates were precleared with protein A-Sepharose beads (Amersham) prepared according to the manufacturer's instructions. Beads were washed as above, and the proteins bound to the beads were eluted by boiling in 2× SDS sample buffer.

Constructs. myc-CHES1 was described previously (27). CHES1ΔDBD cDNA was obtained from t223 clone (25) and subcloned into CS2MT vector to create myc-CHES1ΔDBD. To generate glutathione S-transferase (GST)-MEN1, human MEN1 cDNA was cut out of pGEM-T-hMEN1 vector and inserted into EcoRI site of pGEX-4T-1 vector (Pharmacia, Piscataway, NJ). To generate MEN1Δ1, pGEX-MEN1 was digested with BamHI and KpnI and relinked in-frame using the following linkers: CLrink (gattt) and BMHlink (gatcagacgta). To generate MEN1Δ2 and MEN1Δ3, pGEX-MEN1 was digested with Eagl and relinked. There are two Eagl sites in human MEN1 cDNA, and fragments of two different lengths were obtained after ligation. To generate MEN1Δ4, pGEX-MEN1 was digested with KpnI and NotI. The ends were blunted with T4 polymerase (New England Biolabs, Ipswich, MA) and ligated.

GST pull-down assay. To generate GST or GST-MEN1 bound agarose beads, BL21 chemically competent Escherichia coli (Stratagene, La Jolla, CA) was transfected with pGEX, pGEX-MEN1, or pGEX-MEN1Δ1-4 vector. One colony from each transfection was picked the next day and grown overnight in 10 mL Luria-Bertani (LB) medium supplemented with ampicillin. Overnight culture (1 mL) was expanded into 50 mL of LB/ampicillin for 2 hours, and then 50 μL of 100 mmol/L isopropyl-thio-B-d-galactopyranoside was added to induce protein synthesis for 4 hours. Bacteria were pelleted by centrifugation and washed with 5 mL of cold STE buffer [0.1 mol/L NaCl, 10 mmol/L Tris-HCl (pH 8), 1 mmol/L EDTA]. Pellets were lysed in 5 mL of STE buffer supplemented with 5 mmol/L DTT, 100 μg/mL lysozyme, complete protease inhibitors, and 70 μL Triton X-100. Lysates were incubated on ice for 10 minutes, vortexed for 2 minutes, and centrifuged (10,000 rpm, 10 minutes, 4°C). Supernatants (protein extracts) were incubated overnight at 4°C with 1 mL GST agarose (Amersham) prepared according to the manufacturer's instructions. Beads were washed four times with 10 mL of cold PBS and resuspended in 1 mL of cold PBS.

CHES1 or CHES1ΔDBD protein was prepared by in vitro translation using Wheat Germ TsT kit (Promega, Madison, WI) according to the manufacturer's instructions. The presence of CHES1 or CHES1ΔDBD after translation was accessed by Western blot analysis.

In vitro–translated CHES1 or CHES1ΔDBD (75 μL) was incubated with GST, GST-MEN1, or GST-MEN1Δ1-4 bound beads for 4 hours at 4°C. Beads were washed as above, and the proteins bound to the beads were eluted by boiling in 2× SDS sample buffer.

Results

Mnn1 mutant Drosophila has defective checkpoint response to DNA damage. We previously reported that the viability of Mnn1 mutant flies is decreased after irradiating radiation exposure compared with WT. However, Drosophila Mnn1 mutants seemed to have normal G2 arrest after irradiating radiation exposure as evaluated by phosphorylated histone H3 staining in larval wing imaginal discs (Fig. 1A; ref. 22). We did a time course of G2-M arrest and reentry into mitosis following radiation exposure in mutants and WT. The dynamics of G2-M arrest in Mnn1 mutant flies were indistinguishable from that of WT Drosophila tissue in both wing (Fig. 1B) and eye (data not shown) imaginal discs. These data suggest that Mnn1 function is not required for proper G2-M arrest after DNA damage.

To examine the S-phase arrest, we assessed BrdUrd incorporation in larval brains before exposure and 1 hour after the exposure to 4 krad of ionizing radiation. WT Drosophila tissue exhibited a marked decrease in DNA synthesis 1 hour after irradiating radiation (Fig. 2A and B), but in Mnn1 mutants, this arrest was incomplete (Fig. 2C and D). A similar defect was described previously for fly mutants of Drosophila ATR and Chk1 homologues. Both of these genes are essential for checkpoint control after DNA damage (28).

MEN1-deficient MEFS fail to undergo S-phase arrest after irradiating radiation. To determine if MEN1 function in DNA damage response is conserved in mammalian cells, we used a recently developed Men1−/− cell line (21). Similar to Mnn1-deficient Drosophila (22), Men1−/− mutant MEFS were hypersensitive to irradiating radiation treatment (Fig. 3A). Incorporation of BrdUrd was markedly decreased in WT MEFS 12 hours after irradiating radiation but persisted in Men1−/− null cells (Fig. 3B and C). In WT, only 4% of cells were still incorporating BrdUrd after irradiating radiation (compared with 20% before treatment). In Men1−/−, 23% of cells were still in S phase (compared with 30% before treatment; Fig. 3C).

Nucleotide incorporation may represent both DNA replication and repair synthesis. To access the cell cycle profile of Men1 mutant MEFS in a manner independent of nucleotide incorporation, we measured DNA content by propidium iodide staining followed by fluorescence-activated cell sorting (FACS) analysis...
radiation treatment varied significantly from WT with much of cells in G2 (Fig. 3) continuing through the S phase, which leads to an accumulation of Men1 intact in mutant cells, these cells are inappropriately reentry into mitosis were similar in mutants and WT.

A genetic screen for Mnn1 interactors identified transcription factor CHES1. Overexpression of Mnn1 in the Drosophila thorax results in viable flies with a thoracic cleft phenotype. This phenotype can be modified by co-overexpression of several known interactors of MEN1, such as components of Drosophila AP-1 transcription factor Jun and Fos. To identify novel potential genetic interactors of Mnn1, we did a screen aimed at isolating genes that modify this thoracic cleft phenotype when coexpressed with Mnn1. One of the interactors isolated by this screen was a member of forkhead/winged helix transcription factor family, Ches1.

Ches1 is a novel member of a large family of forkhead/winged helix transcription factors, many of which were implicated in cancer (27). The human homologue of Ches1, CHES1, was reported to be down-regulated in several tumors and cancer cell lines (29, 30). Both CHES1 and menin were reported to bind Sin3a (11, 31), and it is possible that they could be in the same complex in a cell. Finally, human CHES1 could rescue viability and restore DNA damage-induced G2 arrest when overexpressed in Saccharomyces cerevisiae checkpoint mutants (25). Thus, the interaction of MEN1 and CHES1 may provide a link between the role of MEN1 in transcription regulation and DNA damage response.

**Ches1 restores S-phase arrest in Mnn1 mutant Drosophila.** Because CHES1 was able to restore DNA damage-induced G2 arrest and viability in several yeast checkpoint mutant strains, including yeast ATR mutant mec-1 (25), we decided to investigate whether overexpression of Drosophila Ches1 in the Mnn1 mutant background could restore G2-S arrest. Flies that were mutant for Mnn1 and overexpressed Ches1 under the control of the actin promoter (ubiquitous expression) were viable and had no detectable developmental or morphologic abnormalities (data not shown). The S-phase arrest in these animals was restored to WT levels (Fig. 2E and F). In addition, overexpression of Ches1 restored the viability of Mnn1 mutants after ionizing radiation exposure to the level of Mnn1 heterozygotes (data not shown).

In yeast, mutations in RPD3 or SIN3 have been shown to compensate for loss of the G2-M checkpoint in mec-1 (ATR) and rad9 (S3BP1) mutant strains. This effect was not enhanced by simultaneous CHES1 overexpression. In addition, overexpression of SIN3 suppressed CHES1-mediated G2-M arrest, providing strong evidence that CHES1 acts through antagonism of HDAC effects (31). We hypothesized that a similar mechanism might be taking place for Ches1-induced restoration of viability and S-phase arrest of Mnn1 mutant flies. If this were true, decreased HDAC activity in Mnn1 mutants would be expected to rescue cell cycle arrest and viability after ionizing radiation exposure. However, in Drosophila, haploinsufficiency for Rpd3 alone caused increased ionizing radiation sensitivity (data not shown), suggesting that this function of Rpd3 may not be conserved in flies. It is possible that Ches1 acts through either other HDACs or through a different mechanism to restore the DNA damage checkpoint in Drosophila Mnn1 mutants.

**Human menin and CHES1 biochemically interact.** To evaluate a possible biochemical interaction between menin and CHES1, we used mammalian reagents. We cotransfected HEK293 cells with the plasmid carrying human MEN1 cDNA under the control of a cytomegalovirus promoter and either full-length myc-CHES1, NH2-terminally truncated myc-CHES1ΔDBD (Fig. 4A), or an empty myc-tag vector. CHES1 or CHES1ΔDBD was immunoprecipitated from cell lysates with α-myc antibody. The α-myc antibody brought down only when CHES1 was used but not when CHES1ΔDBD or empty vector was cotransfected with menin (Fig. 4B). In the converse experiment, we immunoprecipitated menin from the cell lysates with α-menin antibody and assessed whether CHES1 communoprecipitated with it. Full-length CHES1, but not CHES1ΔDBD, could be communoprecipitated with menin (Fig. 4C). These results suggest that menin and CHES1 are in the same complex or might directly interact and that the NH2-terminal portion of CHES1 containing the DNA-binding domain is necessary for this interaction.

To investigate if menin could directly bind CHES1, we used a GST pull-down assay. Glutathione beads bound to GST-menin or GST alone were incubated with in vitro–translated CHES1 or CHES1ΔDBD proteins. In agreement with our communoprecipitation results, only full-length CHES1 was found to bind GST-menin (Fig. 5A and B). Although these experiments confirm the

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immunoprecipitation data, we cannot rule out a possibility that additional proteins contained in the in vitro translation mix form a complex with CHES1 and menin, in which the two proteins of interest do not interact directly.

To determine which part of menin protein is required for CHES1 binding, we generated four truncation constructs of \textit{MEN1} cDNA cloned into a GST vector. Full-length menin protein contains 610 amino acids. The truncation mutant MEN1\textsubscript{D1} contained amino acids 241 to 610, MEN1\textsubscript{D2} contained amino acids 1 to 241, MEN1\textsubscript{D3} contained amino acids 1 to 353, and MEN1\textsubscript{D4} contained amino acids 1 to 428 (Fig. 5C). Only the NH₂-terminal truncation mutant MEN1\textsubscript{D1} retained the ability to bind CHES1 (Fig. 5D), whereas MEN1\textsubscript{D2-4} mutants, all having lost the COOH-terminal part of menin, were no longer able to bind CHES1. These results suggest that the CHES1-binding domain of MEN1 is located between amino acids 428 and 610 of human menin.

**Discussion**

Multiple lines of evidence exist to support the involvement of menin in transcriptional control and chromatin modification. Human menin was found to bind and control the activity of multiple transcription factors. Menin interacts with mSin3a, a component of Sin3/Rpd3 HDAC complex (11), and with a histone methyltransferase complex containing trithorax family proteins MLL2 and Ash2L (12–15). In addition, menin was found to bind to the promoter regions of several homeobox genes and to regulate their expression (12, 13). Recently, two other transcription targets of menin were identified. Cyclin-dependent kinase inhibitors p27\textsuperscript{kip1} and p18\textsuperscript{ink4c} are down-regulated in menin-deficient cells, which also exhibited increased proliferation (14). Thus, menin may control cell proliferation through regulating transcription of genes involved in cell cycle control.

\textit{MEN1} was also implicated in the DNA damage response. Our studies in \textit{Drosophila} revealed that flies mutant for \textit{Mnn1} are hypersensitive to ionizing radiation and hypermutable (22). In the present work, we found that \textit{Drosophila Mnn1} mutants as well as \textit{MEN1}-deficient MEFs fail to undergo proper G₁-S arrest in response to ionizing radiation exposure. At the same time, G₂-M checkpoint is unaffected in both fly and mammalian \textit{MEN1} mutant tissue. These data clearly show the existence of functional homology between fly and mammalian menin. The question remains, however, whether \textit{MEN1} is involved in the DNA damage response through its involvement in transcription regulation and chromatin modification.

Chromatin remodeling has been implicated in both regulation of transcription and DNA repair (32). Post-translational modifications of histones by acetylation and phosphorylation as well as nucleosome remodeling by helicases were shown to affect accessibility of damaged DNA to the repair machinery (33–37).
Recently, a SWI/SNF-related nucleosome remodeling complex of *S. cerevisiae*, Ino80, was shown to be directly recruited to double-strand break sites where it facilitated the repair process (38, 39).

The forkhead transcription factor CHES1 may serve as a potential link between transcription control, chromatin modification, and the DNA damage response function of MEN1. CHES1 was identified in a screen for genes that suppress mutagen sensitivity of rad9 (53BP1), dun1, mec-1 (ATR), and rad53 (Chk1) mutant strains of *S. cerevisiae* by restoring UV-induced and ionizing radiation–induced G2 arrest (25). It was further shown that this action of CHES1 required binding to the Sin3/Rpd3 HDAC complex. Inactivation of Sin3 or Rpd3 in rad9 and mec-1 mutant strains also restored cell cycle arrest after DNA damage (31). Thus, altering chromatin structure by changing histone acetylation affected the response to DNA damage stimuli. The function of CHES1 is not well understood. The COOH-terminal domain of CHES1 can act as a transcriptional repressor and interacts with transcriptional regulator protein SKIP (27). In addition, expression studies revealed down-regulation of CHES1 expression in several tumors and tumor cell lines (29, 30).

In this work, we show that *Drosophila* Ches1 can modify the effects of *Mnn1* loss of function, suggesting an *in vivo* genetic interaction between these genes. Because both CHES1 and menin were shown to interact with HDACs and menin was shown to act through HDAC function to repress transcriptional activity of JunD (10), it is possible that CHES1 competes with menin for binding to HDACs and through this mechanism can partially suppress the effects of *Mnn1* overexpression in *Drosophila* thorax. In support of this hypothesis, we previously found that haploinsufficiency for one of the HDACs (Sir2) could partially suppress *Mnn1*-induced split thorax, although Rpd3 did not have similar effect (data not shown).

Overexpression of *Ches1* under the control of the actin promoter restored S-phase checkpoint arrest and viability of *Mnn1*-null *Drosophila* mutants after the exposure to ionizing radiation. In this

![Figure 3. Men1+/− MEFs are deficient in G1-S arrest after ionizing radiation. A, Men1+/− MEFs are hypersensitive to ionizing radiation. Cells were irradiated with indicated doses, and colonies were counted 7 days after ionizing radiation. The experiment was repeated thrice. Bars, SE. Men1+/− MEFs show significantly reduced survival compared with WT at 500 rad (P < 0.03) and at 1,000 rad (P < 0.002). B, fluorescent microscopy analysis of BrdUrd incorporation in WT and MEN1 mutant MEFs. Cells were allowed to incorporate BrdUrd for 15 minutes 12 hours after ionizing radiation and stained with anti-BrdUrd antibody (green) and DAPI (blue) to visualize nuclei. C, FACS analysis of BrdUrd incorporation in WT and MEN1 mutant MEFs before radiation and 12 hours after ionizing radiation. Percentages of cells in each phase are indicated. As in (B), the number of BrdUrd-incorporating cells decreased dramatically in WT but not in MEN1 mutant cells after the ionizing radiation treatment. D, DNA content analysis by propidium iodide staining of WT and MEN1 mutant MEFs before radiation and 12 hours after ionizing radiation. Percentages of cells in each phase are indicated. The distribution of the irradiated MEN1 mutant cells differs significantly from the profile of WT cells due to the lack of G1-S checkpoint.](http://www.aacrjournals.org/doi/fig/3)
case, however, unlike restoration of $G_2$ arrest in yeast checkpoint mutants, Ches1 does not seem to be functioning through HDAC complexes. This result suggests that Ches1 acts through a different mechanism for $G_1$-$S$ and $G_2$-$M$ arrest after DNA damage. Drosophila $ATR$ and $Chk1$ mutants are deficient for both $G_1$-$S$ and $G_2$-$M$ checkpoints (28). However, $Mnn1$ seems to be completely dispensable for $G_2$-$M$ arrest. Thus, $Mnn1$-independent $G_2$-$M$ checkpoint and $Mnn1$-dependent $G_1$-$S$ checkpoint are likely to operate downstream of $ATR$ and $Chk1$.

The ability of Ches1 overexpression to modify the effects of $Mnn1$-null mutants suggests that Ches1 acts downstream of $Mnn1$ in the Drosophila DNA damage response. Because $Mnn1$ mutants used in this work completely lack any protein product from this gene, it is possible that overexpression of Ches1 can substitute for the function of $Mnn1$ in DNA damage response. Alternatively, overexpressing Ches1 may activate a secondary pathway able to bypass the requirement for $Mnn1$ in $S$-phase arrest. However, taking into account the biochemical interaction between human menin and Ches1, we favor the hypothesis that $MEN1$ and Ches1 act in the same pathway.

In support of the genetic interaction between $Mnn1$ and Ches1 in Drosophila, we found that human menin biochemically

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**Figure 4.** Human menin and human Ches1 coimmunoprecipitate. A, schematic representation of full-length Ches1 and its truncated version, Ches1ΔDBD, myc-tag attached at NH2 terminus. Light gray, part of Ches1 binding to yeast Sin3 (amino acids 292-490; ref. 31); dark gray, SKIP-binding domain (amino acids 424-490; ref. 27). B, HEK293 cells were transfected with indicated constructs. Lysates were immunoprecipitated (IP) with α-myc antibody and probed with α-menin antibody. Menin coimmunoprecipitated with full-length Ches1, but not with Ches1ΔDBD, or when cotransfected with empty myc-tag vector. Membranes were stripped and reprobed with α-myc antibody to ensure the presence of Ches1 and Ches1ΔDBD in the lysates (data not shown). C, experiment was done as in (B), except that lysates were immunoprecipitated with α-menin antibody and probed with α-myc antibody. Full-length Ches1, but not Ches1ΔDBD, coimmunoprecipitated with menin. Membranes were stripped and reprobed with α-menin antibody (data not shown).

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**Figure 5.** Menin binds Ches1 in GST pull-down assays. Full-length in vitro–translated Ches1 (A) or Ches1ΔDBD (B) protein was incubated with GST or GST-MEN1, and complexes were revealed by Western blot. Full-length Ches1, but not Ches1ΔDBD, bound to GST-MEN1. C, schematic representation of full-length MEN1 protein and four truncated MEN1 constructs. JunD-interacting domains (amino acids 1-40, 139-242, and 323-448; ref. 6); mSin3a-interacting domain (amino acids 371-387; ref. 11). D, in vitro–translated Ches1 protein was incubated with GST, GST-MEN1, or any of the four MEN1 truncation mutants. None of the three COOH terminally truncated mutants bound Ches1, whereas NH2 terminally truncated GST-MEN1Δ1 was still able to bind Ches1. The presence of GST-MEN1 and GST-MEN1Δ1 was assessed by anti-menin antibody, the presence of GST, and GST-MEN1Δ2-4 was assessed with anti-GST antibody (data not shown).
interacts with human CHES1. The CHES1 COOH-terminal domain containing amino acids 293 to 490 and lacking the DNA-binding forkhead domain was shown to interact with both SKIP and yeast Sin3 (27, 31). For menin, however, the COOH-terminal domain of CHES1 was insufficient for binding and only full-length protein could interact with menin. This raises the possibility that CHES1 may be bound to menin and other factors at the same time, bringing several proteins together in a complex. In addition, we discovered that the COOH terminus of menin (amino acids 428-610) is required for binding to CHES1.

Many mutations of the MEN1 gene in MEN1 patients affect this part of the protein (http://www.hgmd.cf.ac.uk/). This suggests the possibility that the formation of the complex containing menin and CHES1 might be involved in MEN1-related tumor formation.

Based on the data presented here, we suggest that menin acts in the regulation of transcription as well as in the S-phase checkpoint control after DNA damage possibly through a mechanism involving modulation of chromatin architecture in cooperation with CHES1 and histone modifying complexes.

**References**

MEN1 Interacts with CHES1 in DNA Damage Response

In the article on how MEN1 interacts with CHES1 in DNA damage response in the September 1, 2006 issue of Cancer Research (1), a footnote should have been included indicating that Valeria Busygina and Molly C. Kottemann contributed equally to the work.


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